The Porcine Cervix

Morphological changes and infiltration of immune cells during oestrus and dioestrus in the sow

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ABSTRACT

During the oestrous cycle, the porcine cervix undergoes marked macroscopic changes: at oestrus the cervix is firm, and after termination of oestrus it progressively softens. However, little is known about the parallel microscopic changes in the cervix. Therefore, the aim of this study was to examine the histological properties of the epithelium and subepithelial connective tissue of the uterine cervix during oestrus and dioestrus; the morphology of the epithelium and the infiltration of cells of the immune system were described. In addition, the proliferative activities of the epithelium and subepithelial connective tissues were investigated by use of an immunohistochemical method that identifies a nuclear protein (Ki-67) present in proliferating cells only. Six sows, three in oestrus and three in dioestrus, were used for this study.

The cervical epithelium was found to vary between simple columnar, pseudostratified and stratified, with mainly stratified epithelium being found at oestrus and mainly simple columnar at dioestrus. No cyclic variations in the number of immune cells in the epithelium of subepithelial connective tissue could be found. Neither could the proliferative activity be proved to change with different stages of the oestrus cycle.

SAMMANFATTNING

Cervix hos gris genomgår stora makroskopiska förändringar under östralcykeln: under östrus är cervix fast i konsistensen medan den mjuknar då östrus övergår i diöstrus. Det finns dock endast lite kunskap om de samtidiga mikroskopiska förändringarna i cervix. Syftet med denna studie var därför att undersöka de histologiska egenskaperna hos epitel och subepitelial bindväv i den uterina delen av cervix under östrus och diöstrus, med avseende på morfologiska förändringar i epitelet och infiltration av immunceller. Dessutom undersöktes förekomsten av proliferation i epitel och subepitelial bindväv, genom att ett nukleärt protein (Ki-67) som endast förekommer i prolifererande celler, identifierades med hjälp av en immunohistokemisk metod. I den här studien ingick sex suggor, tre i östrus och tre i diöstrus.

Epitelet i cervix varierade mellan enkelt cylinderepitel, flerradigt och flerskiktat epitel. Under östrus fanns huvudsakligen flerskiktat epitel och under diöstrus framför allt enkelt cylinderepitel. Vecken i slemhinnan var tjockare under östrus än diöstrus, vilket tyder på ödem i vävnaden vid östrus. Inga cykliska variation i antalet immunceller i epitelet eller i den subepiteliala bindväven kunde påvisas. Inte heller kunde det bevisas att förekomsten av proliferation varierar mellan östrus och diöstrus.

INTRODUCTION

During the oestrous cycle, the porcine cervix undergoes thorough changes in consistency that can be identified by rectal palpation in sows (Meredith, 1977; Kunavongkrit et al., 1983). However, little is known about the morphological changes of the cervix during the oestrous cycle. The cervix also marks the border between the uterus and the outside world, especially during pregnancy, and it is not unlikely that it participates in the protection of the uterus from intruding microorganisms, for example also during coitus, when the boar's penis may bring in infectious agents. Hence it is of interest to investigate the presence of cells of the immune system in the cervix.

The aim of this study was therefore to investigate the histological properties of the cervix during two stages of the oestrous cycle (oestrus and dioestrus). The cervical epithelium and subepithelial connective tissue as well as the infiltration of cells of the immune system are described. The results are compared to descriptions of morphological changes in other species and other parts of the porcine tubular reproductive organs.

In an attempt to further clarify the cyclic changes in the cervical tissue, the proliferative activities of the cervical epithelium and subepithelial connective tissues during oestrus and dioestrus are investigated; this is done by use of an immunohistochemical method that identifies a nuclear protein (Ki-67) present in proliferating cells only. Ki-67 is expressed in G1, S, M and G2 phase, but absent in G0 (i.e. in resting cells) (Scholtzen & Gerdes, 2000).

REPRODUCTION IN THE FEMALE PIG

The female reproductive organs in the pig consist of the ovaries, oviducts, uterus, cervix, vagina, vestibulum and vulva, of which all except the ovaries constitute the tubular genitalia.

Reproductive physiology in the sow

Puberty and sexual maturation

The gilt reaches puberty at the age of 6-7 months, but the exact time is influenced by breed, management, nutritional factors and season. The onset of puberty and the subsequent physiological changes are governed by the activity of the ovaries, which in turn are controlled by the hypothalamus-pituitary. In particular, the luteinizing hormone (LH) is involved in the onset of ovarian activity.

The oestrous cycle

The domestic pig is considered to be poly-oestral, meaning that the sow or gilt has regular oestrous cycles throughout the year, except when pregnant or lactating. The oestrous cycle is divided into several phases: prooestrus (1-3 days), oestrus

(1-3 days), metoestrus (2-3 days) and dioestrus (13-18 days). The first day of standing oestrus is generally considered to be the first day of the cycle.

In average, the length of the oestrous cycle is 21 days (18-24 days). Prooestrus and oestrus are referred to as the follicular phase, as follicles are the predominant structures in the ovaries, whereas metoestrus and dioestrus are collectively called the luteal phase, with the corpora lutea being the main functional ovarian structures during this phase. In the pig, around 20 follicles ovulate each oestrous cycle, giving rise to a corresponding number of corpora lutea. Pregnancy lasts approximately 113 days. During lactation, the female pig is usually in anoestrus. The interval between weaning and oestrus is 4-6 days.

Endocrinology of the oestrous cycle

The oestrous cycle is controlled by the hypothalamic-pituitary-ovarian axis. Other parts of the brain also influence cyclic activity, mediating the effect of light (season), smell, touch etc. In addition, the uterus exerts an effect on the cycle by secreting substances such as prostaglandin that affect ovarian activity. The hypothalamus controls the pituitary by releasing GnRH (gonadotropin releasing hormone) which stimulates the pituitary to secrete hormones (FSH and LH) that, in turn, stimulate follicular growth in the ovaries.

In response to stimulation by FSH (follicle stimulating hormone), growing follicles secrete oestradiol (oestrogen) that further stimulates follicular growth and acts on the uterus, cervix and outer genitalia in preparation for mating and reception of the fertilised ova. Oestrogen causes the typical signs of oestrus such as a swollen and hyperaemic vulva, restlessness and riding behaviour as well as an increase in secretory activity, hypertrophy and oedema of the reproductive tract. The rising oestrogen level stimulates a release of LH (luteinizing hormone) from the pituitary in a peak-like fashion, which in turn initiates the process of ovulation. Ovulation occurs at the beginning of the last third of the oestrus phase.

During oestrus, the sow or gilt is sexually receptive. She will assume a rigid posture (standing reflex) when her loins are firmly pressed, either by a boar or by human manipulation in the presence of a boar, and she will accept mating. Due to the influence of oestrogen, there is oedema of the endometrium and cervix and an increase in the secretion of mucus.

During metoestrus, cells in the ruptured follicles are converted to lutein cells, which will form the progesterone-producing corpora lutea. The oestrogen level rapidly declines, followed by an increase in the level of progesterone.

During dioestrus, the corpora lutea continue to produce progesterone that acts on the uterus to create favourable conditions for reception of the embryo and placentation and stimulate the uterine glands to increase their secretory activity. In the case of fertilisation and pregnancy, the corpora lutea will continue the production of progesterone until shortly prior to parturition, and will also start producing relaxin. Progesterone exerts a negative feedback effect on the hypothalamus, inhibiting GnRH-release and thus the final maturation and ovulation of new follicles.

In the absence of pregnancy, the corpora lutea will regress towards the end of dioestrus due to the influence of PGF-2α (prostaglandin) secreted from the non-pregnant uterus. The PGF-2α leaves the uterus with the venous blood and passes directly to the ovary by diffusion between the uterine vein and the ovarian artery – an arrangement made possible by the close contact between the two vessels. As the corpora lutea regress the level of progesterone decreases which results in the removal of the inhibition on the hypothalamus. This enables release of GnRH and the development of new follicles, and thus a new cycle commences.

Short summary of events during the oestrous cycle

Prooestrus (1-3 days):

- follicular growth and regression of the corpora lutea of the previous cycle
- rising oestrogen level from the developing follicles leads to an increase in secretory activity and to the typical outer signs of an approaching oestrus

Oestrus (1-3 days):

- the sow or gilt is sexually receptive and shows a standing reflex
- oestrogen causes oedema of the oviducts, endometrium, cervix and vulva and an increase in the secretion of mucus
- ovulation occurs

Metoestrus (2-3 days):

- formation of corpora lutea
- declining oestrogen level and rising progesterone level

Dioestrus (13-18 days)

- active progesterone-producing corpora lutea
- progesterone stimulates secretion from the uterine glands
- the uterus is prepared for fetal membrane attachment and placentation
- luteolysis occurs towards the end of dioestrus, ending the production of progesterone unless vital embryos are present

Anatomy and histology of the porcine female reproductive organs

As mentioned above, the female reproductive organs in the pig consist of the ovaries, oviducts, uterus, cervix, vagina, vestibulum and vulva. The histology of the tubular genitalia follows a general pattern. Facing the lumen is the mucosa, consisting of an epithelium with properties differing between organs, and a connective tissue stroma of varying depth and structure. Other types of cells, such as immune cells, are also found in the connective tissue layer. Outside the mucosa is the muscularis which contains two, more or less distinct, layers of smooth muscle cells, a circular inner layer and a longitudinal outer layer. Surrounding the muscularis is a thin layer of connective tissue. In the peritoneal cavity the organs are covered by a serosa consisting of a simple squamous epithelium (the peritoneum) and in the pelvic cavity an adventitia of loose connective tissue.

The ovaries

The ovaries in the pig are approximately 5 cm long and irregular in shape, due to numerous follicles and/or corpora lutea protruding from the surface in cyclic animals. The ovaries have two principal functions: storage and development of oocytes (egg cells), and production of endocrine sex hormones. The oocytes are

contained within follicles in different stages of development – primary, secondary and antral follicles. Antral follicles may undergo further development into mature follicles that ovulate. The ovaries are suspended from the abdominal roof by long mesovaria. The histology of the ovaries will not be discussed further.

The oviducts

The oviducts, or uterine tubes, are about 20 cm in length and divided into three segments. Facing the ovary is the infundibulum, which is designed to capture the egg at ovulation. It has a funnel-shaped open end towards the ovary with a fringe of small projections called fimbriae that are in contact with the ovary. The oviducts then consist of the wider ampulla and the narrower isthmus, which connects to the uterus. The ampulla-isthmus junction is the site of fertilisation, i.e. where the spermatozoa meet the ova. The oviducts are attached to the peritoneal wall by the mesosalpinx, which is a continuation of the mesovarium.

The oviductal mucosa consists of a simple columnar or pseudostratified epithelium with ciliated and secretory cells, the cilia stroking towards the uterus. Underneath the epithelium is a layer of loose connective tissue which is greatly folded, particularly in the infundibulum and ampulla. Two layers of smooth muscle cells surround the mucosa. Outermost is a thin layer of connective tissue covered by a serosa.

The uterus

The pig uterus consists of a short (3-4 cm) body and two long uterine horns (*corpus* and *cornuae uteri*). The length of each cornua is approximately 60 cm in gilts, 100 cm in a non-pregnant sow and may reach 200 cm in the pregnant sow. The uterus, oviducts and ovaries are located in the abdominal cavity and are suspended from the abdominal roof by the broad ligaments (*ligamentum latae*), including the mesometrium, continuing as mesosalpinx and mesovarium.

The uterine mucosa is referred to as the endometrium and consists of a simple to pseudostratified columnar epithelium with ciliated and secretory cells, and a layer of loose connective tissue containing coiled tubular glands. The subepithelial layer is richly vascularised and infiltrated by several types of cells of the immune system. The myometrium (muscularis) has two layers of smooth muscle, an inner circular layer, and an outer longitudinal layer. The myometrium thickens during pregnancy. The outer layer (perimetrium) is, like in the oviducts, made up of a layer of connective tissue covered by a serosa.

The cervix

The cervix is mainly located in the pelvic cavity. It is up to 25 cm in length and has internal interdigitating mucosal prominences (*pulvini cervicales*). The cervix lacks distinct ends; in particular the transition between cervix and vagina is continuous. The utero-cervical transition is somewhat more obvious.

In the sow, the cervical epithelium consists of a mix of columnar cells and stratified squamous cells, which can cover more than 90 % of the mucosa. The mucosa forms folds which can sometimes be mistaken for glands. The muscularis consists of inner circular and outer longitudinal layers. The layers of smooth

muscle tend to be arranged in bundles surrounded by connective tissue and the muscularis does not extend into the mucosal prominences (Rigby, 1967).

The vagina, vestibulum and vulva

The vagina reaches from the cervix to the urethral orifice and the vestibulum reaches from the urethral orifice to the vulva. The vulva is composed of the labia and the clitoris. The epithelium in the vagina and vestibulum is of the stratified squamous type. The vestibulum in particular has subepithelial lymphatic nodules in the connective tissue stroma. Except for the most cranial part of the vagina, that has a serosa, these parts of the reproductive tract are contained within the pelvic cavity and therefore surrounded by a tunica adventitia of loose connective tissue.

Cyclic changes in the tubular genitalia of the sow

The oviducts

A study by Jiwakanon et al. (2005) showed that at prooestrus and oestrus, the epithelium in the ampulla and infundibulum was high columnar and pseudostratified with a high degree of mitotic activity and secretory granules. At dioestrus, the epithelium was low columnar with some degree of pseudostratification, and much less secretory granules and mitotic activity. The isthmus showed a lower degree of morphological changes than the ampulla and infundibulum during all stages of the oestrous cycle. The cyclic changes of the porcine oviduct have been described in another study (Abe & Oikawa, 1992) as well, where similarly, more profound cyclic changes were found in the ampulla and infundibulum than in the isthmus. It has also been shown that the degree of submucosal oedema was highest at oestrus, when the plasma oestrogen level is high (Jiwakanon et al., 2005).

According to Jiwakanon et al. (2005) the lymphocyte was the dominating immune cell in the oviductal epithelium. In the submucosa the lymphocyte and the plasma cell were the most common immune cell types, but low numbers of neutrophils, macrophages, mast cells and eosinophils were also found. The number of intraepithelial lymphocytes and macrophages did not differ between oviductal segments or oestrous cycle stages, whereas the number of lymphocytes, plasma cells and neutrophils in the submucosa differed significantly between different parts of the oviduct, but not between different stages of the cycle.

The uterus

The morphological properties of the endometrium vary during the oestrous cycle. Kaeoket et al. (2002) found that during oestrus and early dioestrus, the epithelium was high columnar and pseudostratified, changing into low columnar at dioestrus, and simple cuboidal or low columnar at late dioestrus and prooestrus. In an earlier study by Stroband et al. (1986) no significant variations in epithelial height were seen during the oestrous cycle, but during oestrus, pseudostratification of the uterine epithelium and a high number of mitoses were noted. According to Kaeoket et al. (2002), the mitotic activity in the epithelium was highest at prooestrus and oestrus, whereas the secretory activity in the uterine glands was highest at dioestrus. During late dioestrus, prooestrus and oestrus there was uterine oedema in the subepithelial connective tissue.

The same study also described the infiltration of immune cells in the endometrium throughout the oestrous cycle. They found that the lymphocyte was the most common immune cell in the endometrium, and that the infiltration of immune cells changed with the cycle stages. In the surface epithelium there were lymphocytes mainly at oestrus and early dioestrus, and macrophages at prooestrus and oestrus.

In the submucosa, the dominating cell type during all stages of the oestrous cycle was the lymphocyte, with the highest numbers of cells being found during oestrus and early dioestrus. At prooestrus and oestrus, there was also a massive infiltration of neutrophils in the submucosa, while during the other stages neutrophils were rare. In accordance with these results, Stroband et al. (1986) also found large numbers of polymorphonuclear leukocytes during oestrus. Mast cells, macrophages, plasma cells and eosinophils were seen in the endometrium; with plasma cells most commonly found in the glandular layer, where macrophages and lymphocytes were seen as well (Kaeoket et al., 2002). Oestrous cycle stage significantly influenced the number of lymphocytes, neutrophils, eosinophils and plasma cells.

The cervix

The cervix is subject to radical macroscopic changes during the oestrous cycle. At the approach of oestrus the cervix becomes increasingly firm and projects horizontally into the abdominal cavity (Meredith, 1977). After oestrus, it progressively softens to hang limply over the pubic brim by day 7 of the oestrous cycle.

There is little information to be found on variations in the histological properties of the cervix during the oestrous cycle. In one study, Steinbach & Smidt (1970) could not find any significant cyclic variation in the height of the epithelium of the uterine part of the cervix.

The vagina and vestibulum

It has been shown that the height of the epithelium of the vagina undergoes significant cyclic variation with maximum thickness in late prooestrus (Steinbach & Smidt, 1970).

The porcine cervix

The porcine cervix can be divided into two distinct portions: the uterine portion which is 4-5 cm in length, and the vaginal portion which is about 10-12 cm (Smith & Nalbandov, 1958). One major difference between the two parts is that the uterine cervix remains closed, i.e. contracted, throughout the oestrous cycle. The vaginal part on the other hand undergoes cyclic closure and relaxation.

Changes during the oestrous cycle

Smith & Nalbandov (1958) found that the cervix was most constricted during oestrus. According to their study, constriction was at its maximum at day 1 and 2 of the oestrous cycle, after which the cervix relaxed gradually until day 9 of the

cycle. Constriction then gradually increased from day 13-14. Rigby (1967) found that the cervical prominences (*pulvini cervicales*) were firm during oestrus and soft after the end of oestrus.

The prominences in the uterine part of the cervix fit together more closely than those in the vaginal part (Rigby, 1967). The lumen of the uterine cervix does not change in size during the oestrous cycle, but the uterine part of the cervix is more easily distended during dioestrus. The firmness of the cervix during oestrus seems to be due to oedema, and not to vascular engorgement or muscular constriction. The change in cervical consistency therefore appears to be caused by softening and hardening of the tissue rather than to constriction and relaxation.

The increase in firmness of the cervix occurs parallel to the increase in plasma oestrogen level that precedes oestrus, which indicate that oestrogen is involved in the change of consistency of the cervix (Kunavongkrit et al., 1983). Smith & Nalbandov (1958) showed that ovariectomy causes complete and sustained relaxation of the vaginal portion of the cervix, but has no apparent effect on the uterine portion. They also demonstrated that injection of oestrogen in ovariectomized sows caused the cervix to constrict, whereas withdrawal of oestrogen resulted in cervical dilatation.

Kunavongkrit et al. (1983) found that softening of the cervix during the post-oestrus phase occurred simultaneously to an increase in plasma progesterone. However, progesterone could not cause relaxation of the cervix, neither in ovariectomized sows given oestrogen, nor in sows in heat (Smith & Nalbandov, 1958). It therefore seems likely that softening of the cervix is due to the absence of oestrogen rather than to the presence of progesterone as suggested by both Smith & Nalbandov (1958) and Kunavongkrit et al. (1983).

During pregnancy

The cervix is also subject to radical changes during pregnancy and parturition. In the pregnant pig the cervix serves to protect the uterus, while, at parturition it must be extensible enough to allow the passage of the foetuses. In the pregnant pig it was shown (Eldridge-White et al., 1989) that extensibility and lumen diameter were less for the uterine portion of the cervix than for the vaginal part throughout most of pregnancy, which indicates that the uterine cervix probably is more important in the protection of the uterus than the vaginal part. After day 80, however, the uterine cervix increased its softness and extensibility, so that at term there were no differences between the cervical segments.

The changes in physical properties of the uterine cervix are correlated with elevated serum oestrogen and relaxin levels. Relaxin and oestrogen levels increased from about day 80 until term, whereas progesterone levels were elevated throughout pregnancy (Eldridge-White et al., 1989). Progesterone and relaxin are produced by the corpora lutea, and oestrogen is produced by the placenta.

It has been shown that in ovariectomized pregnant gilts, relaxin is important in causing softening and growth of the cervix prior to parturition (O'Day et al., 1989). Progesterone alone, administered to maintain pregnancy, could not

increase the extensibility of the cervix. It was also shown that oestrogen alone did not increase extensibility of the cervix after day 80 of pregnancy. Relaxin influenced compositional changes in cervical connective tissue, such as a decrease in collagen concentration and an increase in water content, dry weight and GAG/collagen ratio (O'Day-Bowman et al., 1991). Relaxin has been shown to be an important cause of histological changes in the pig cervix during late pregnancy by promoting a reduction in density of collagen and influencing the organization of collagen fibre bundles as well as smooth muscle fibre bundles (Winn et al., 1993).

Winn et al. (1994) found that relaxin alone promoted growth and softening of the cervix in ovariectomized non-pregnant gilts, and that this effect was markedly augmented by progesterone. This study also showed that oestrogen did not influence relaxin's effect on the cervix, which contradicts results from a study by Hall & Anthony (1993) showing that the effect of relaxin on the cervix was enhanced by oestrogen. However, both studies agreed that oestrogen contributes independently to the growth of the cervix (Hall & Anthony, 1993; Winn et al., 1994). Oestrogen is capable of inducing many compositional changes in the cervix, such as increased water content, and altered collagen and GAG concentrations, but these changes do not result in increased cervical distensibility (Hall & Anthony, 1993).

MATERIALS AND METHODS

Animals

For this study six crossbred sows (Swedish Landrace × Swedish Yorkshire) were used. The animals were purchased from a commercial herd and brought directly after weaning to the Division of Reproduction, Department of Clinical Sciences (former Department of Obstetrics and Gynaecology) at SLU (the Swedish University of Agricultural Sciences). The sows had an average parity number of 4 (range 3-5). Body weight ranged from 185-268 kg. All sows had previously shown normal reproductive performance. The sows were housed in individual pens with boars housed in the same stable. They were fed the Swedish standard diet for dry sows (Simonsson, 1994) and had water available ad libitum.

The sows were controlled twice daily for signs of oestrus by inspection of vulva for reddening and swelling, and by controlling the standing reflex in the presence of a boar. Sows slaughtered during the oestrus period were controlled every 4 hours from the onset of prooestrus. In each sow, ovarian follicular development and ovulation were followed by transrectal ultrasonography (Kaeoket et al., 2002).

Blood and tissue sampling

Blood was collected from the jugular vein one hour prior to slaughter for determination of levels of plasma oestradiol 17β (E_2) and plasma progesterone (P_4) by immunoassay techniques, as described by Kaeoket et al., (2002).

Three sows were slaughtered at oestrus (day 1 of the cycle) and three sows were slaughtered at dioestrus (day 11-12). The animals were stunned and bled and the internal genital organs were removed immediately. Tissue samples for light microscopy and immunohistochemistry were collected from the uterine part of the cervix, within 5 cm from the uterus. Samples for morphological evaluation by light microscopy were fixed in 3% glutaraldehyde in 0.067 M sodium cacodylate buffer (pH 7.4). Samples for immunohistochemistry were fixed in 5 % paraformaldehyde.

Morphology

Preparation of tissue

The tissue samples were trimmed, dehydrated and embedded in water soluble methylmetacrylate (Historesin, LKB, Bromma, Sweden). Semi-thin sections (2 μm) were obtained with a rotation microtome (Leica RM 2165), placed on glass slides and stained with buffered toluidine blue.

Morphological evaluation

Microscopic evaluation of the samples was carried out by one person (Karin Edström). All slides were coded before examination, to ensure that the identity of the animals would not be known. For morphological examination a light microscope (Leitz Laborlux 12) was used, with objective 40× and eyepiece 12.5×. One eyepiece was equipped with a reticule of 100 small squares, arranged so that 10 small squares made up one side of a larger square. In a magnification of 12.5×40, the side of one small square corresponded to 0.03 mm of tissue and the small square had an area of 0.0009 mm^2.

Only tissue areas free of artefacts were used for counting. Due to technical problems, it was difficult to obtain sections of acceptable quality taken a fixed distance apart. Therefore two slides from each animal were examined and the results are presented as two series.

All photographs were taken with a Nikon Microphot-FXA.

Epithelium

10 lengths of epithelium, each length consisting of 10 small squares, were examined. In total a length of 100 small squares equalling 3 mm of epithelium was examined in each slide. For each length, the epithelium was examined on the following parameters:

- description of the epithelium as simple/stratified/pseudostratified, columnar/squamous and presence of cilia and secretory cells
- number of intraepithelial lymphocytes (located near the basal membrane), macrophages and neutrophils

Subepithelial connective tissue

In each section, 5 areas consisting of 20 small squares each were counted (100 small squares in total). A depth of 2 squares (0.06 mm) from the basement membrane into the subepithelial stroma was examined. Thus, each examined area was 2×10 squares or 0.06×0.3 mm (0.018 mm^2). For each slide, a total area of 100

small squares, corresponding to a real tissue area of 0.09 mm^2 of subepithelial stroma was examined. The following parameters were counted:

- number of fibroblasts and fibrocytes
- number of small vessels (capillaries, arterioles, venules)
- number of lymphocytes, plasma cells, macrophages, neutrophils and mast cells
- size and lymphocyte density of subepithelial lymphatic assemblies

Immunohistochemistry

The immunohistochemical method used for detection of the Ki-67 protein was an ABC technique, in which an unlabelled primary antibody binds to antigenic sites in the tissue, followed by a biotinylated secondary antibody (binding to the primary antibody), and a horseradish peroxidase-avidin-biotin complex. A kit, containing blocking serum (normal horse serum), a biotinylated secondary antibody (horse-antimouse) and an ABC-complex was used. Three negative controls were obtained by treating one (K2) with normal horse serum only, and two (K1 a+b) with normal mouse IgG. K1b-controls were also stained with haematoxylin.

Preparation of tissue

The fixed tissue was embedded in paraffin, cut into 4 µm thick sections, mounted on polylysine glass slides and dried in room temperature.

Immunohistochemical procedure

The slides were dried overnight in 37°C, deparaffinised in xylene 3×5 min, thereafter rehydrated in successively lower concentrations of alcohol (alcohol abs 1× + 1×2 min; 95% 1× + 1×2 min; 70% 1×2 min) and finally in destH_2O 2×5 min. To unmask antigenic sites, the slides were boiled in citrate buffer (0.01 M Na-citrate buffer, pH 6.0) in a pressure cooker (2100 Retriever, Prestige Medical) for 20 min and were allowed to cool down for 40 min followed by washing in PBS buffer for 2×5 min [phosphate-buffered saline, pH 7.4; stock solution: dissolve 80 g NaCl, 2 g KCl, 10.6 g Na_2HPO_4, 2 g KH_2PO_4 in 500 ml destH_2O; adjust pH to 7.4; before use the stock solution was diluted 20×with destH_2O]. Endogenous peroxidase activity was blocked with 3% H_2O_2 (hydrogen peroxidase) in methanol (10 ml 30% H_2O_2 + 90 ml methanol) in darkness for 10 min. The slides were then washed in PBS buffer for 2×5 min.

Before incubation with the primary antibody, the slides were pretreated with normal horse serum (14 µl NHS + 934 µl PBS) for 30 min in a dark moist chamber at room temperature. The primary antibody to Ki-67 (MM1, Novocastra Laboratories Ltd, United Kingdom, diluted 1:200 in PBS) was added, except to K2 where the normal horse serum was left and K1 a+b, to which Mouse IgG (sc-2025, Santa Cruz Biotechnology Inc, USA, diluted 1:100 in PBS) was added. All slides were left to incubate for 2 h in a dark moist chamber at room temperature.

After primary antibody binding, all slides were washed in PBS-buffer for 2×5 min and then incubated with the secondary antibody (horse-anti-mouse, Vectastain PK 6102 Elite, Vector Laboratories Inc, USA, diluted 1:200 in PBS) for 30 min in a

dark moist chamber at room temperature. After renewed washing in PBS buffer for 2×5 min, the ABC-complex (Vectastain PK 6102 Elite, Vector Laboratories Inc, USA, diluted 1:50 in PBS) was added, the slides were incubated in a dark moist chamber at room temperature and then washed in PBS buffer for 2×5 min. The slides were treated with a colour substrate (DAB kit, Saveen Biotech, 25 mg/ml in 100 ml PBS + 75 µl H_2O_2) in darkness for 8 min and then washed in destH_2O 5× + 2×5 min. All slides except K1a and K2 were counterstained with haematoxylin for 15 s and then rinsed in running tap water for 15 min. After dehydration with successively higher concentrations of alcohol (70% 1×2 min; 95% 1×2 min; alcohol abs 1×2 min) and xylene 1×2 min, the slides were mounted with permanent mounting medium (Pertex, Histolab Products AB, Gothenburg, Sweden) and dried in room temperature.

Immunohistochemical evaluation

Evaluation of the samples was carried out by one person. All slides were coded before examination, to ensure that the identity of the animals would not be known. Only tissue areas free of artefacts were used for counting. A light microscope was used, with objective, eyepiece and reticule as described above.

In the epithelium, a total tissue length of 0.3 mm was examined, from 10 different locations, each 10 small squares long. In the subepithelial connective tissue a total tissue area of 0.09 mm^2 was examined, from 5 different locations, each consisting of 2×10 small squares. The number of cells with moderate and strong positive immunostaining for Ki-67 was counted. In the epithelium, only positive cells located near the basal membrane were counted, due to the varying types of epithelium. The controls were examined for absence of staining.

As one of the slides was damaged in the immunohistochemical process, a corresponding slide from an earlier immunohistochemical experiment was used instead. In order to ensure that the two experimental series were comparable, two slides from another animal from each of the experimental sessions were examined.

Statistical analysis

Statistical analysis was done using the SAS statistical package (SAS Institute Inc., 1989). One-way analysis of variance was performed on the data from the cell counts and the immunohistochemical evaluation of Ki-67 using the general linear model (GLM) procedure.

RESULTS

Hormone levels

The blood levels of oestradiol and progesterone at oestrus and dioestrus are presented in figure 1. The plasma level of oestradiol was high at oestrus and low at dioestrus, while the plasma level of progesterone was low at oestrus and high at dioestrus.

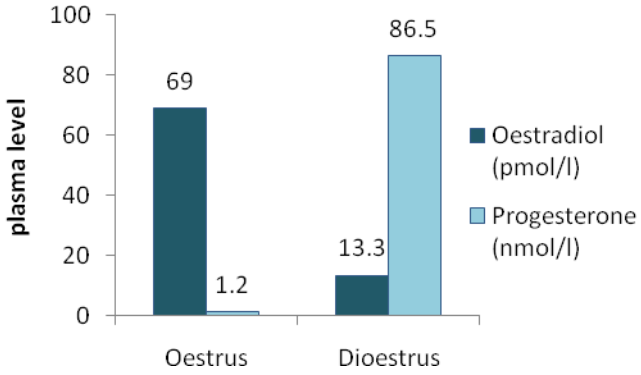


Figure 1. The mean plasma levels of oestradiol (pmol/l) and progesterone (nmol/l) at oestrus and dioestrus.

Morphology

General findings

The mucosa was folded to a varying extent. In general, the folds appeared to be thicker in animals in the oestrus phase, than in animals in the dioestrus phase. In the oestrus animals, primary and secondary folds were seen, whereas in the dioestrus animals, primary, secondary and tertiary folds were seen (see figure 2).

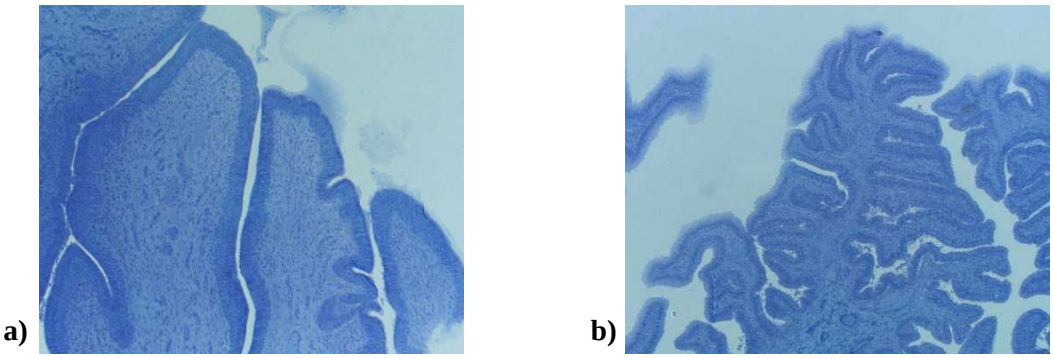


Figure 2. Folding of the cervical mucosa at a) oestrus and b) dioestrus

Epithelium

Description of the epithelium

The epithelium in the uterine cervix was found to be of a varying character. In most slides, several types of epithelium could be identified. The epithelium varied between simple columnar, pseudostratified columnar, basally stratified with columnar cells facing the lumen and stratified squamous. The columnar cells were either ciliated or appeared to be secretory. In some animals, the type of epithelium changed frequently, resulting in a "patchy" appearance (see figure 4). In animals in the oestrus phase, the epithelium was mainly stratified, whereas at dioestrus the epithelium was mostly simple columnar (see figure 3).

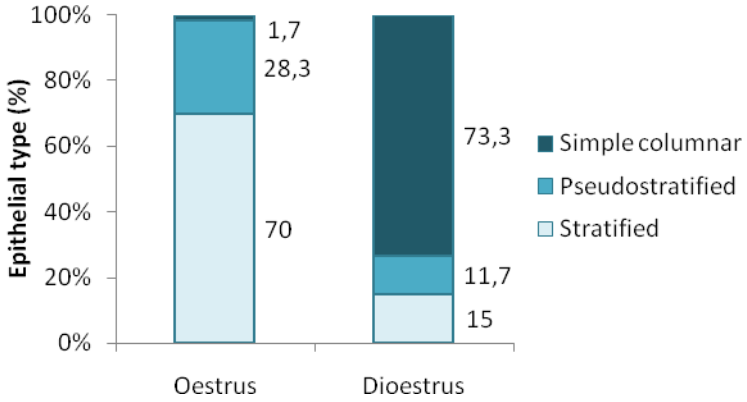
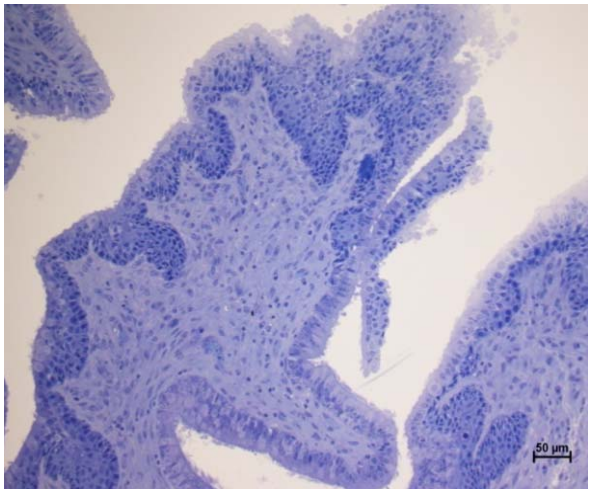


Figure 3. Distribution (%) of different types of epithelium in the uterine cervix at oestrus and dioestrus.

Figure 4. Transition between different types of epithelia in the porcine cervix:

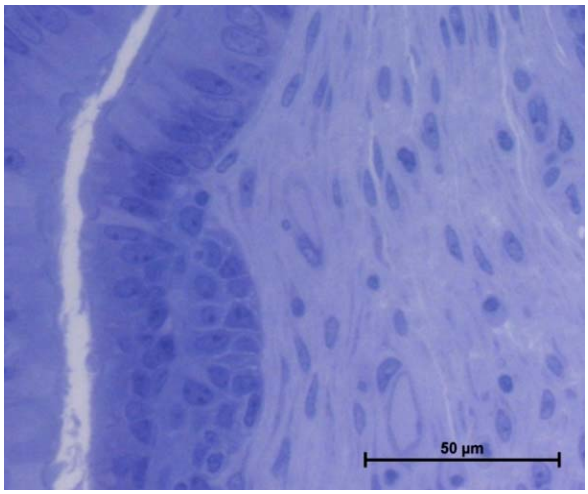
a) a fold of cervical mucosa with simple columnar and stratified epithelium

a)



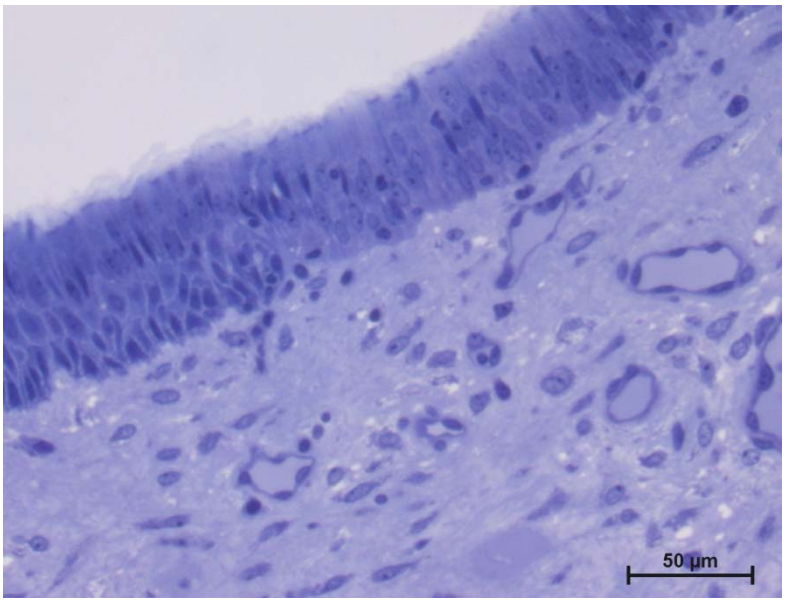
b) transition from simple columnar into a patch of stratified epithelium

b)



c) pseudostratified epithelium changing into a stratified epithelium with columnar cells facing the lumen

c)



Intraepithelial immune cells

The results from the counting of intraepithelial immune cells are presented in table 1 as numbers of lymphocytes and macrophages/100 small squares of epithelium (3 mm). Neutrophils were also looked for, but none were found.

Table 1.The number of intraepithelial lymphocytes and macrophages /100 small squares length in the uterine cervix; mean value and range for each cell type and series

Cell type	*Series*	**Oestrus**	**Dioestrus**
Lymphocytes	Series 1	45.3 (31-57)	50.7 (33-73)
	Series 2	41 (23-52)	40.7 (30-52)
Macrophages	Series 1	6.3 (4-11)	0.7 (0-2)
	Series 2	1.7 (0-3)	0.3 (0-1)

The lymphocyte was found to be the most common immune cell in the epithelium of the uterine cervix. The number of cells could not be found to vary between the two stages of the oestrus cycle, although there was a tendency for more macrophages being present at oestrus.

Subepithelial connective tissue

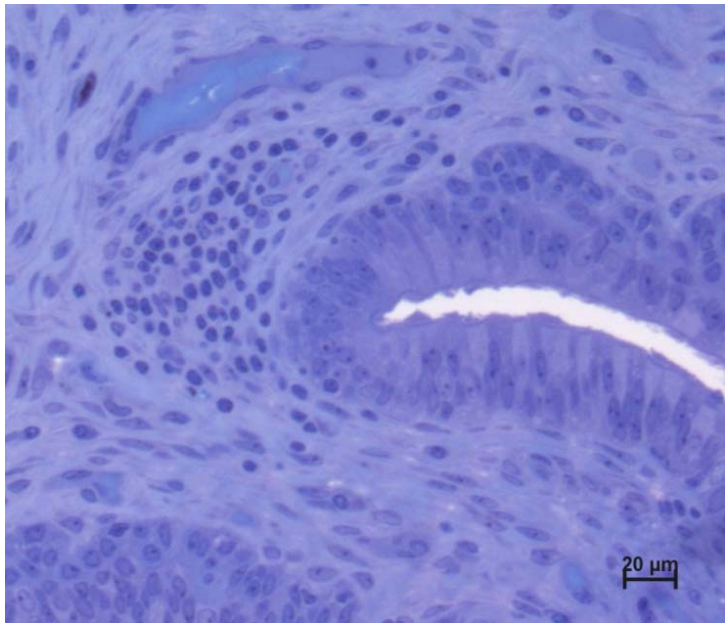
In the subepithelial connective tissue, the number of fibroblasts, vessels and cells of the immune system were counted. In addition, the size and lymphocyte density of subepithelial lymphatic assemblies were estimated.

Subepithelial lymphocyte aggregations

In three animals, subepithelial aggregations of lymphocytes were noted (see figure 5). The size varied between 9 to 30 small squares (0.0081 to 0.027 mm^2) and the

lymphocyte density varied between 3.8 to 15 cells/small square (0.0009 mm^2) with a mean of 8.5 cells/small square.

Figure 5. Subepithelial aggregation of lymphocytes



Fibroblasts and vessels

The number of fibroblasts and vessels was counted and the result is summarized in table 2. The result is presented as number of cells/tissue area of 100 small squares. No significant variations in the number of fibroblasts or vessels were found.

Table 2. Number of fibroblasts and vessels /100 small squares in the subepithelial connective tissue (to a depth of 0.06 mm from the basal lamina), mean value and range for each cell type/structure and series

Cell type	*Series*	**Oestrus**	**Dioestrus**
Fibroblasts	Series 1	208.3 (152-261)	175.3 (169-187)
	Series 2	183.7 (129-248)	165 (145-179)
Vessels	Series 1	22.7 (21-26)	24.3 (19-32)
	Series 2	19.7 (16-22)	21 (17-23)

Immune cells

The number of immune cells was counted and the result is summarized in table 3. The result is presented as number of cells per tissue area of 100 small squares. As can be determined from the data, the lymphocyte was found to be the most common cell in the subepithelial connective tissue of the uterine cervix, followed by the plasma cell. Macrophages appeared to be somewhat more common than mast cells and neutrophils. The majority of neutrophils were seen in the lumen of small vessels. No significant differences in the numbers of immune cells in the

subepithelial connective tissue could be found between oestrus and dioestrus animals, although lymphocytes, macrophages and mast cells appeared to be present in higher numbers during oestrus.

Table 3. Numbers of immune cells /100 small squares in the subepithelial connective tissue (to a depth of 0.06 mm from the basal lamina)

Cell type	*Series*	**Oestrus**	**Dioestrus**
Lymphocytes	Series 1	79.3 (17-130)	45 (33-61)
	Series 2	69.7 (44-104)	50.7 (48-54)
Macrophages	Series 1	2 (0-3)	2.7 (0-5)
	Series 2	2.7 (1-5)	3.3 (1-5)
Plasma cells	Series 1	28 (20-41)	21.7 (17-24)
	Series 2	22.7 (11-37)	31.3 (25-43)
Mast cells	Series 1	1.3 (0-2)	0 (0)
	Series 2	0.7 (0-2)	0 (0)
Neutrophils	Series 1	1.7 (1-3)	1 (0-2)
	Series 2	1 (0-2)	1 (0-2)

Immunohistochemistry

Epithelium

The counting of immunopositive cells is presented as the number of strongly and moderately stained cells per an epithelial length of 100 small squares (corresponding to a real tissue length of 3 mm). The numbers of epithelial cells immunopositive for Ki-67 are summarized in table 4. The observations initially seemed to suggest a higher degree of proliferative activity in the cervical epithelium during the oestrus phase, but the tendency could not be shown to be statistically significant. Examples of positively stained cells are shown in figure 6.

Table 4. The number of Ki-67 immunopositive cells (strongly and moderately stained) /100 small squares length in the epithelium of the uterine cervix during oestrus and dioestrus

Staining intensity	**Oestrus**	**Dioestrus**
Strong	47.7 (28-78)	16.7 (14-20)
Moderate	56 (31-105)	19 (8-30)
Total	103.7 (28-105)	35.7 (8-30)

Subepithelial connective tissue

The result is presented as the number of strongly and moderately stained cells per tissue area of 100 small squares (corresponding to a real tissue area of 0.09 mm^2). Figure 6 shows examples of positively stained cells. The numbers of Ki-67 immunopositive cells in the subepithelial connective tissue are summarized in

table 5. Also here, the results initially gave an impression of higher proliferative activity during oestrus, but as was the case for the epithelium the difference was not statistically significant.

Table 5.The number of Ki-67 immunopositive cells (strongly and moderately stained) /100 small squares in the subepithelial connective tissue in the uterine cervix during oestrus and dioestrus

Staining intensity	**Oestrus**	**Dioestrus**
Strong	18 (12-26)	5.3 (2-8)
Moderate	15.3 (6-28)	3 (1-7)
Total	33.3 (6-28)	8.3 (1-8)

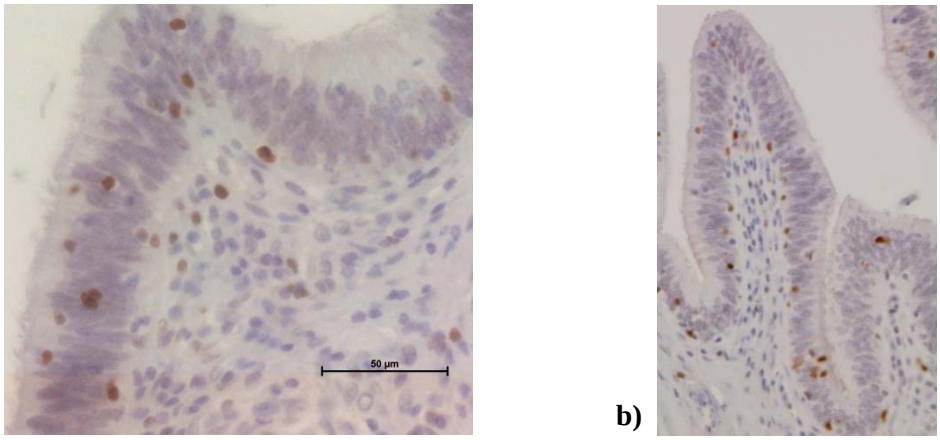


Figure 6 a) and b). Ki-67 immunopositive cells in the epithelium and subepithelial connective tissue of the porcine cervix at oestrus.

DISCUSSION

Since only sparse information has been found on cervical morphology in the pig, comparison with results from earlier studies is not possible. Instead, the morphology of the cervix throughout the oestrous cycle will be related to the conditions in other parts of the reproductive tract, compared to other species and discussed in relation to function.

Epithelium

Morphology

Observations made during this study indicate that the epithelium of the uterine part of the cervix is of a varying character, changing between stratified, pseudostratified and simple columnar, and that there are a number of ciliated and secretory cells present. The stratified epithelium was often found to have a layer of columnar cells facing the lumen. A similar phenomenon has also been described for the mouse (Corbeil et al., 1985), where the cervical epithelium can

consist of a layer of cuboidal cells overlying a layer of stratified squamous cells. However, the porcine cervix differs from the murine cervix in that a layer of keratinized cells may be found under the cuboidal surface cells at prooestrus and oestrus in the mouse, whereas no cornified cells have been found in the porcine cervix in this study.

One aim of this study was to examine the cervical epithelium for evidence of cyclic variations. The epithelium was found to be mainly stratified at oestrus, and mainly simple columnar at dioestrus (see figure 3). This suggests a cyclic variation of the cervical epithelium in the pig. In the mouse, it has been found that the location of the junction between squamous and columnar epithelium in the cervix shifts during the oestrous cycle, so that at prooestrus/oestrus it is located closer the vagina, at early metoestrus closer to the uterus and at dioestrus it is quite indistinct (Corbeil et al., 1985).

It is reasonable to believe that the character of the epithelium changes from a columnar type closer to the uterus, into a more stratified type closer to the vagina, since a stratified epithelium is more resistant to abrasion. In mice, cell size and shape in the reproductive tract change gradually from the uterine end, where cells tend to be rounder and smaller, to the vaginal end, where cells are broader and flatter (Corbeil et al., 1985).

Thus, according to these observations the cervical epithelium appears to be subject to cyclic variations, although these variations are different in character from what is seen in, for example, the oviduct (Abe & Oikawa, 1992; Jiwakanon et al., 2005) and the uterus (Kaeoket et al., 2002). In the oviduct and uterus, variations in height and degree of pseudostratification occur in the columnar epithelium, whereas the cervical epithelium appears to change character from simple columnar into stratified. This seems likely, since a more resistant mucosa is needed at oestrus, and a stratified epithelium is less sensitive to possible damage inflicted by the boar's penis at coitus.

However, it should be kept in mind that it is difficult to ensure that the cervical tissue is taken from exactly the same location in all animals, since the uterine cervix of the pig is rather short and lacks distinct borders. In addition, no clear transition between simple and stratified epithelium seem to exist in the porcine cervix, as a "patchy" type of epithelium is frequently found. Thus, it cannot be said for certain whether any cyclic changes actually occur, or whether the apparent differences are due to individual variation regardless of oestrous cycle stage.

The immunohistochemical study of the nuclear protein Ki-67 failed to show a higher degree of proliferative activity during oestrus than during dioestrus, although at first, the numbers seemed to indicate that. Further studies and larger groups of animals would be needed to clarify whether a difference exists in the proliferative activity. If indeed the epithelium change from simple columnar at dioestrus into a stratified at oestrus, a higher degree of proliferation at some point is likely.

Immune cells

The lymphocyte was found to be the most common immune cell in the epithelium of the uterine cervix (summarised in figure 7, see also table 1). The number of intraepithelial lymphocytes and macrophages do not seem to show any cyclic alterations since no significant differences in the number of cells could be found between the stages.

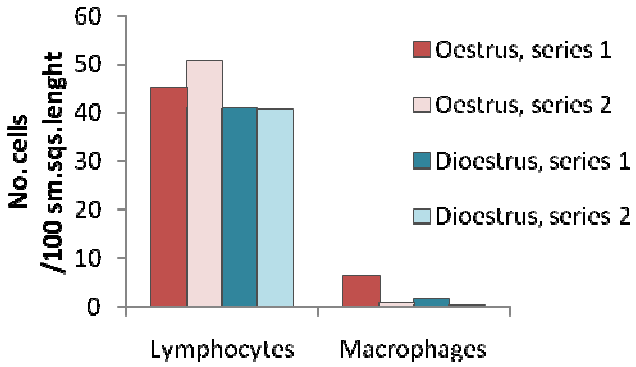


Figure 7. Distribution of intraepithelial lymphocytes and macrophages in the epithelium of the uterine cervix.

The lack of cyclic variation is in accordance with the situation described for the oviduct, where the number of intraepithelial lymphocytes and macrophages did not differ between different stages of the oestrous cycle (Jiwakanon et al., 2005). In the uterus, however, observations have shown that lymphocytes in the epithelium are present in higher numbers at oestrus and early dioestrus, and macrophages at prooestrus and oestrus (Kaeoket et al., 2002).

Subepithelial connective tissue

Morphology

As was the case for the epithelium, no significant differences in proliferative activity in the subepithelial connective tissue could be found between oestrus and dioestrus, even though the data initially seemed to suggest otherwise. Few animals in the experimental groups and a high individual variation can be the explanation.

The folding of the mucosa was more extensive during dioestrus than oestrus, with primary, secondary and tertiary folds at dioestrus and only primary and secondary folds at oestrus, which suggests that a degree of oedema is present during oestrus. However, no effect on cellular density in the subepithelial connective tissue could be noted, since the amount of fibroblasts and vessels did not vary between the two oestrous cycle stages.

According to Rigby (1967) the increase in firmness of the cervix during oestrus is due to oedema causing hardening of the tissue. It seems likely that the events leading to the increase in consistency take place deeper into the cervical

connective tissue. This would explain why no impact on cellular density has been found in this study.

Immune cells

The distribution of immune cells in the subepithelial connective tissue is summarised in figure 8 (see also table 3). As in the uterus (Kaeoket et al., 2002) and oviduct (Jiwakanon et al., 2005), the most common immune cell in the subepithelial connective tissue was the lymphocyte. The high infiltration of neutrophils that occurred in the uterus at prooestrus and oestrus could not been observed in the cervix.

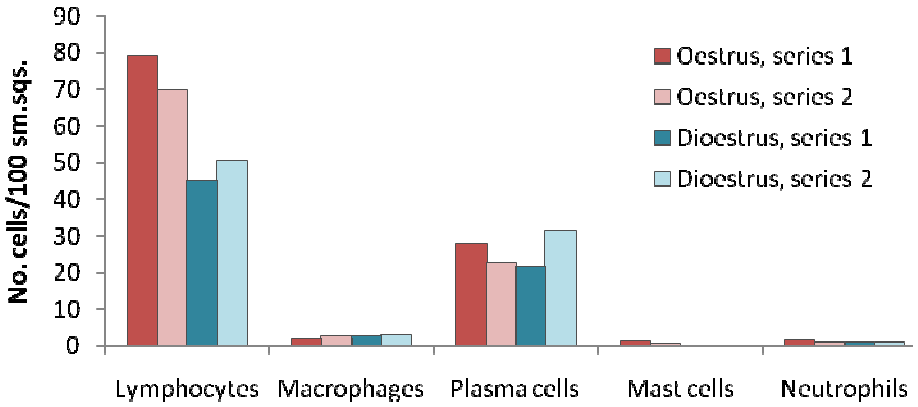


Figure 8. Distribution of immune cells in the subepithelial connective tissue of the uterine cervix.

The infiltration of immune cells could not be shown to vary significantly between the two stages of the oestrous cycle, which differed from the situation in the uterus (Kaeoket et al., 2002) where a massive invasion of neutrophils was found during prooestrus and oestrus, in addition to an increase in the number of other inflammatory cells. In the pig, during mating and artificial insemination, the semen is deposited straight into the uterus (Rath et al., 2008). Therefore, the semen will probably not elicit any major inflammatory reaction in the cervix. It is also likely that the uterus, rather than the cervix, must maintain a higher level of defence against incoming infections during this period.

In ruminants on the other hand, semen is deposited in the vagina close to the cervix. In ewes, high numbers of neutrophils have been found in the vagina and cervical os at oestrus but only low numbers in the mid and anterior cervix (Scott et al., 2006). Following insemination, neutrophils accumulate in the cervical lumen but despite this, only minimal infiltration of neutrophils in the tissue of the mid- and anterior cervix of the ewe has been noted, suggesting migration of neutrophils from the uterus.

In conclusion, the epithelium of the porcine cervix uteri seems to show some degree of morphological variation during the oestrous cycle, but no variations in proliferative activity could be proven statistically. The infiltration of cells of the immune system into the epithelium and subepithelial connective tissue appears to

remain constant throughout the cycle, suggesting that the porcine cervix does not have a need for an elevated defence against microorganisms or reaction to semen. However, in order to clarify the cyclic events in the porcine cervix, more extensive studies are required, including several oestrus cycle stages, larger groups of animals and several samples taken along the cervix.

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